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Immunoneutralisation of GnRH-I, without cross-reactivity to GnRH-II, in the development of a highly specific anti-fertility vaccine for clinical and veterinary use☆

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Abstract

In recent years, several forms of gonadotrophin releasing hormone (GnRH) molecules have been isolated from primate brain. These molecules are very similar in sequence and this raises the question of whether previously developed neutralisation vaccines based on GnRH (now termed GnRH-I) would remove other forms of GnRH (namely GnRH-II) as well. As the function of these other molecules has not yet been clearly defined, potential health risks could exist by their ablation. In view of the high sequence homology between the molecules, this paper describes the production of highly specific polyclonal antibodies against GnRH-I and GnRH-II, with negligible cross-reactivity. The ultimate aim of this is to develop an anti-fertility vaccine which does not present any inappropriate side-effects, caused by neutralisation of a GnRH molecule which may or may not be directly involved in reproduction. Several formulations were investigated, based on analogues of the following molecules,

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conjugated to tetanus toxoid:

GnRH-I	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2
GnRH-II	pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH2

The specificity of the antibodies produced was examined, together with effects on fertility and any inappropriate side-effects. Immunostaining of hypothalamic sections was carried out, using the generated antisera, to determine the regional distribution of GnRH-I and GnRH-II neurones, as well as to further evaluate the specifity of the antibodies.

Keywords: Anti-fertility vaccine; GnRH-I; GnRH-II; Immunisation; Neutralisation

1. Introduction

Until fairly recently, it was believed that the hypothalamic decapeptide, gonadotrophin-releasing hormone (GnRH), was the sole controlling factor in causing the release of luteinising hormone and follicle stimulating hormone from the pituitary, in the regulation of mammalian fertility (Sherwood et al., 1993). However, it was known that different forms of GnRH existed in lower vertebrates (Powell et al., 1994; Kasten et al., 1996). In the last few years GnRH peptides with similar sequences have been isolated from higher vertebrate brains as well (Kasten et al., 1996), including primates (Urbanski et al., 1999; Latimer et al., 2000), although clear identification of their physiological roles has not yet been defined.

Active immunisation against GnRH and its analogues (reviewed in Ferro and Stimson, 1999) has a marked physiological effect on fertility, in both male and female animal species (Ferro et al., 1995a; Ferro and Stimson, 1997, 1999). This occurs through immunoneutralisation of GnRH, resulting in ablation of sex steroid levels, arrest in gametogenesis and extreme gonadal atrophy. The use of this type of therapy is in clinical trial (Thau, 1992; Moudgal et al., 1997), with reportedly encouraging results (Talwar et al., 1992; Pal and Talwar, 1995; Talwar, 1997) as well as being under investigation in the veterinary field (Hoskinson et al., 1990). However, a growing concern due to the high sequence homology between GnRH-I and GnRH-II molecules, is that neutralisation of GnRH-I could lead to inadvertant ablation of GnRH-II as well. Since the full function of GnRH-II is unknown, this could result in long-term complications.

The primary purpose of this study was to selectively produce an antiserum to GnRH-I, with minimal cross-reactivity to GnRH-II. In a preliminary experiment, GnRH-I and GnRH-II peptides, truncated by two amino acids at the N-terminal (des-2), were conjugated to tetanus toxoid and used to immunise male rats over a 10-week period. Specific antibody levels and

cross-reactivity, were assessed by enzyme linked immunosorbant assay (ELISA) (Ferro et al., 1996). Having demonstrated that cross-reactivity between the two forms of GnRH was minimal, the antisera were used to selectively stain primate hypothalamic sections, in order to investigate a physiological role for GnRH-II.

The main aim of this study was to develop a vaccine to control mammalian gonadal function based on a highly specific antibody response to GnRH-I. Therefore, in order to improve the efficacy of immunocastration, another truncated sequence of GnRH-I (des-1, removal of one amino acid at the N terminal) was examined in the rat model. This sequence was further modified with the addition of a cysteine residue at the N terminal and an amide group at the C terminal. Antibody specificity, cross-reactivity and subclass type were assessed. Testicular measurements (Ferro and Stimson, 1996) were taken throughout the study and testosterone levels assayed in order to assess the efficacy of immunisation on reproductive function.

2. Materials and methods

2.1. Immunogen preparation

2.1.1. Conjugation via carboxylic and amine groups

The peptides (0.2 μmol) conjugated to tetanus toxoid (0.1 μmol), using this method were either des-2 GnRH-I (His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly) or des-2 GnRH-II (His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly). Tetanus toxoid (Connaught, Canada) was dialysed against 0.9% saline, pH 6.3, for 2 h at room temperature. Coupling buffer (10 ml), consisting of 0.1 M NaCl, pH 6.0 was added to the tetanus toxoid, together with 6 mg EDC (ethyl-3-[3-dimethyl amino propyl] carbodiimide hydrochloride) (Perbio, UK) and 16 mg *N*-hydroxy succinimide (Perbio, UK) and left for 15 min at room temperature. Peptide (90% purity, synthesised by Immune Systems, UK) was dissolved in 0.5 ml distilled water and added to the tetanus toxoid. The mixture was incubated in the dark, at room temperature for 3 h. The conjugate was dialysed against 0.9% saline, overnight at 4 °C and the volume measured. Conjugation efficiency and carrier:peptide ratio were determined by setting up a standard curve with free peptide (0.015–1 mg/ml). The A_{280} values were recorded together with a 1:2 dilution of unconjugated tetanus toxoid control. The A_{280} of the conjugate was subtracted from the A_{280} of the control and the resulting absorbance read against the standard curve to calculate the concentration of conjugated peptide. The conjugate was dialysed against flake polyethylene glycol (M_w 20 000, Sigma-Aldrich, UK) for 2 h at room temperature, followed by 2 h against 0.9% saline and then stored frozen, until required for immunisation.

2.1.2. Conjugation via suphydryl and amine groups

The peptide sequence Cys-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2 (des-1 GnRH-I analogue, 2 μmol, 90% purity, synthesised by Immune Systems, UK) was conjugated to tetanus toxoid (0.1 μmol) using sulpho-maleimido benzoic succinimide (S-MBS, Perbio, UK) as described previously (Aithal et al., 1988; Ferro et al., 1996).

2.2. Immunisation schedule

Male Sprague–Dawley rats were housed in a fully climatised room: room temperature 22 °C; relative humidity 55%; light and dark cycles of 12 h each; illumination 60 Lux. In study week 0, at the age of 7 weeks, the rats were randomised, divided into groups of eight and ear-coded.

In the preliminary experiment, the immunogen was emulsified with Freund's complete adjuvant (Sigma-Aldrich, UK), such that each dose consisted of 1 ml of a 1:1 mix of conjugate:adjuvant. The rats were immunised intraperitoneally in study week 1 and booster injections were given in study weeks 2, 3, 4 and 10 with Freund's incomplete adjuvant.

In the subsequent experiment, the following modifications were made. The immunogen (50 μg equivalent of peptide) was mixed in a 1:1 ratio with an aluminium hydroxide based adjuvant, Imject alum (Perbio, UK). The mixture was left for 30 min before use. The animals were immunised intraperitoneally in study weeks 1, 3, 5, 7.

2.3. Antibody estimations

Three days after each immunisation, tail bleeds were carried out into capillary tubes (Hawksley and Sons, UK) and the blood centrifuged at $1000 \times g$ for 20 min to obtain serum for measurement of antibody levels. The sera were frozen until specific ELISAs were carried out on BSA:peptide coated plates as described previously (Ferro et al., 1996).

2.3.1. Indirect assay to measure specific antibody levels

Peptide-BSA conjugate (equivalent to 1 μg peptide/well in 100 μl phosphate buffered saline [PBS], pH 7.4) was coated onto tissue culture grade 96-well plates for 1 h at 37 °C. The plates were washed twice with wash buffer (PBS, containing 0.01% Tween 20) and blocked with 3% (w/v) Marvel in PBS-Tween, for 1 h at 37 °C. The plates were washed three times with wash buffer. Rat sera (0.1 ml, diluted 1:1000 in PBS, prepared from tail bleeds) was incubated per well for 1 h at 37 °C [carried out in triplicate for each sample]. The plates were washed three times with PBS-Tween. Horse radish peroxidase labelled goat-anti-rat IgG (Perbio, UK) was diluted

1:3000 in PBS and 100 μl/well incubated for 45 min at 37 °C. The plates were washed three times with PBS (without Tween) and developed with 0.1 ml TMB substrate/well (250 μl of stock 6 mg/ml 3,3′,5,5′-tetramethyl benzidine in dimethylsulphoxide, added to 25 ml 0.1 M sodium acetate buffer, pH 5.5 with 4 μl 30% (v/v) hydrogen peroxide). The reaction was stopped with 50 μl/well 10% (v/v) sulphuric acid after 15 min and the A_{450} read. The means of the triplicate results were calculated and plotted on a graph against the study week number.

2.3.2. Indirect assay to measure antibody cross-reactivity

ELISAs were carried out, as above, but anti-GnRH-I sera was examined on GnRH-II-BSA-coated plates and vice versa.

2.3.3. Indirect assay to assess antibody subclasses

Peptide specific ELISAs were carried out as described above, but horse radish peroxidase-labelled goat-anti-rat IgM, IgG1-IgG2c (manufactured by Bethyl laboratories USA, distributed by Universal Biologicals, UK), diluted 1:5000 in PBS, were used in place of whole labelled goat anti-rat IgG. Pooled antisera from CHWSYGLRPG-NH2 immunised animals, taken at post-mortem, was used as positive control, with labelled goat-anti-rat whole IgG for the second antibody. The means of triplicate results were plotted on a graph against the study week number.

2.4. Assessment of reproductive physiological effects

Single measurements of combined testicular widths were carried out on a weekly basis throughout the studies. This was found to provide an appropriate observation of the effect of immunisation on reproductive function.

At the end of each study, the animals were exsanguinated by section of the abdominal aorta distal to the renal arteries; the blood was retained for further analyses. Brief autopsies were also performed on the animals to identify any adverse effects of immunisation; the testes, seminal vesicles and epididymes were weighed and then preserved in 10% formalin in PBS.

2.4.1. Histological comparison of the testes and epididymes

The formalin fixed tissues were cut into small pieces (0.5 cm^2), and embedded in 'Tissue Tek' (Raymond A Lamb, UK), at −70 °C. The tissues were sectioned (thickness 6 μm) in a cryotome (Shandon Scientific,

UK), at −15 °C. Finally the sectioned tissues were fixed for 5 min onto silanised superfrost slides (BDH, UK) with Carnoy's fluid (60% Absolute alcohol, 30% chloroform and 10% glacial acetic acid) and stained with Mayer's Haematoxylin and Eosin (prepared as described by Luna, 1968). The slides were dipped for 2 min in the following solutions, unless otherwise stated: 100% alcohol, 100% alcohol, 95% alcohol, tap water, distilled water, Mayer's haematoxylin (25 s), tap water, distilled water, 80% alcohol, Phloxine-Eosine, 95% alcohol, 100% alcohol, 100% alcohol, 100% alcohol:xylene mix (1:1, 30 s), xylene mount DPX.

Morphometric analysis of the testicular sections was performed under bright-field microscopy using a CCD camera and the Image computer program (National Institutes of Health).

2.4.2. Testosterone estimation

Testosterone levels were determined in the serum, prepared from blood obtained at autopsy. A direct competitive radioimmune assay (Euro/DPC, UK) was used according to the manufacturers' instructions.

2.5. Hypothalmic expression of GnRH-I and GnRH-II

In order to help elucidate the physiological function of GnRH-II, comparative immunohistochemistry was performed on paraformaldehyde-fixed hypothalamic sections from rhesus macaques (*Macaca mulatta*), using the anti-GnRH-I and anti-GnRH-II sera from the preliminary study. The sections were prepared as detailed previously (Latimer et al., 2000). Free-floating coronal sections (25 μm) were washed three times, 5 min each, with Tris buffer (0.05 M Tris, pH 7.6, containing 0.15 M sodium chloride). They were then incubated in 2% normal rabbit serum in Tris buffer for 20 min and washed as before. Next, the sections were incubated for 48 h at 4 °C with anti-GnRH-I, anti-GnRH-II, or pre-immune sera at a 1:500 dilution in Tris buffer. They were then washed three times, 5 min each, incubated in biotinylated rabbit anti-rat IgG (Vector Laboratories, Burlingame, CA) at a 1:500 dilution in Tris buffer for 1 h at room temperature, and again washed. To detect the signal, the sections were exposed to an avidin/biotin complex (Standard ABC kit; Vector Laboratories) for 1 h, washed three times in Tris buffer, 5 min each, and exposed to 3,3′-diaminobenzidine tetrachloride (1 mg/ml Tris buffer; Sigma, St. Louis, MO) for 5 min, concluding with three more washes. The sections were then mounted on glass slides (Fisherbrand Superfrost/Plus; Fisher, Auburn, WA) and air dried for 1 h. The slides were then serially dehydrated through ascending ethanol concentrations, dipped in xylenes, and coverslipped with DPX (Electron Microscopy Sciences, Fort Washington, PA).

3. Results

3.1. Efficiency of conjugation

The peptide:carrier ratio for the des-2 GnRH-I to tetanus toxoid or bovine serum albumin, varied between 8:1 and 14:1, with conjugation efficiencies ranging from 40 to 70%. On the other hand, the peptide:carrier ratio for des-2 GnRH-II was consistently lower at 4:1–7:1, with conjugation efficiencies of 20–35%. When using the S-MBS method, the peptide:carrier ratio was 20:1 and 100% efficient.

3.2. Assessment of specific antibody levels

A comparison of the specific antibody levels for des-2 GnRH-I, des-2 GnRH-II and des-1 GnRH-I is shown in Fig. 1. In the des-2 peptide animals (which were immunised in study weeks 1, 2, 3, 4 and 10), IgG levels rose slightly in both groups of animals following the second immunisation, and increased substantially after three administrations. In the des-1 GnRH-I analogue treated animals (which were immunised in study weeks 1, 3, 5, 7), IgG levels rose after the first administration and continued to rise after the subsequent immunisations.

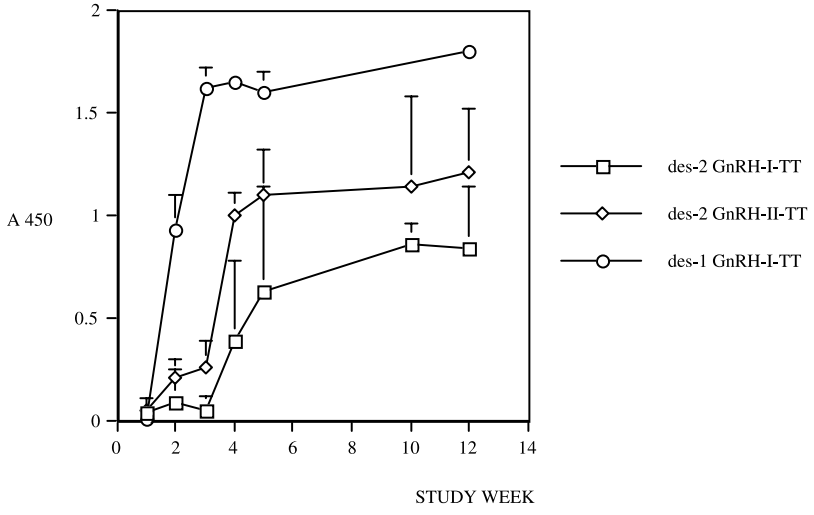


Fig. 1. The IgG antibody response of animals immunised against des-2 GnRH-I, des-2 GnRH-II and des-1 GnRH-I. The A_{450} were measured from specific ELISAs carried out on BSA-peptide coated plates. Immunisations were carried out in study weeks 1, 2, 3, 4, 10 (for the des-2 treated animals) and 1, 3, 5, 7 (for the des-1 treated animals).

Table 1
The assessment of cross-reactivity of antisera from the des-1 (GnRH-I-TT) and des-2 (GnRH-I-TT and GnRH-II-TT) treated animals on ELISA plates coated with the des-2 peptides, conjugated to BSA

Treatment	ELISA Plate coating	
	GnRH-I-BSA $A_{450} \pm$ S.D.	GnRH-II-BSA $A_{450\,nm} \pm$ S.D
des 2 GnRH-I-TT	0.838 ± 0.302	0.211 ± 0.481
des 2 GnRH-II-TT	0.164 ± 0.076	1.206 ± 0.358
des 1 GnRH-I-TT	1.793 ± 0.058	0.000 ± 0.000

Each value represents the mean $\pm$ S.D. of triplicate A_{450} ELISA readings. The antisera tested were from study week 12.

The des-1 GnRH-I analogue treated animals produced the maximum response and levels rose immediately, then plateaued after 2 weeks. On the other hand, both the des-2 analogues (GnRH-I and GnRH-II) showed a 3-week lag, before concentrations rose. Despite the difference in levels, the pattern of response was virtually the same for all treatments.

3.3. Assessment of antibody cross-reactivity

Anti-GnRH-I sera did not cross-react with GnRH-II beyond background levels, and vice versa as shown in Table 1.

Antisera from animals immunised against the des-1 GnRH-I analogue showed no cross-reactivity with GnRH-II. Therefore it was decided to further examine these antisera.

3.4. Antibody subclasses in des1 GnRH-I analogue treated animals

Fig. 2 shows the results from ELISAs used to determine antibody subclasses. The results show the presence of all the subclasses examined. The peak A450 was observed in study weeks 4–5 and the order of magnitude was IgG2c > IgG2a > IgG1 > IgG2b > IgM.

3.5. Testicular width measurements

The des-1 GnRH-I analogue immunised animals showed a significant reduction in combined testicular width, following the third administration, compared with saline-treated animals (Table 2).

3.6. Post-mortem assessment of gonadal atrophy

At post-mortem, five out of eight of the des-2 GnRH-I-TT immunised animals showed a reduction in weight of combined testes, epididymes, prostate and seminal vesicles compared with the non-treatment controls (results not shown). Whereas, none of the des-2 GnRH-II-TT immunised animals showed any gross gonadal changes.

On the other hand, all the animals treated with the tetanus toxoid conjugated des-1 GnRH-I analogue, showed a significant reduction in the size of the reproductive organs (Table 3), compared with untreated controls. These results were attributed to the negligible testosterone concentrations of the treatment group (0.0 ± 0.0 ng/ml compared with the untreated controls (1.63 ± 0.77 ng/ml).

Histologically, the testes in the des-1 GnRH-I analogue treated animals showed marked reduction in spermatogenesis, with azospermia and marked depletion of Leydig cells. Fig. 3a shows a section through an untreated control testis compared with Fig. 3b a section through a des-1 GnRH-I analogue treated animal. Both sections are at the same magnification ($\times 12.5$ objective lens). Marked atrophy of the testis leading to testicular failure was characterised by azoospermia of the seminiferous tubules. The

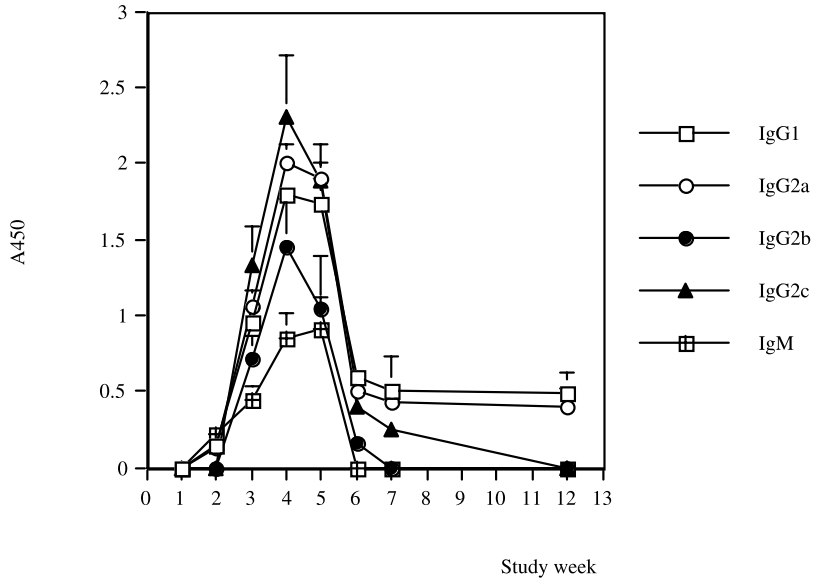


Fig. 2. The IgM and IgG subclass response of animals immunised against the tetanus toxoid conjugate of des-1 GnRH-I. ELISAs were carried out on BSA-des-1 GnRH-I coated plates. Immunisations were administered in study weeks 1, 3, 5, 7.

Table 2
The mean combined testes width at terminal sacrifice in animals immunised against des-1 GnRH I-TT compared with control animals

Study week	Combined testes width (mm) ± S.D.	
	No treatment	des 1 GnRH-I-TT treatment
1	20.04 ± 1.65	19.74 ± 1.16
2	18.03 ± 1.51	18.36 ± 1.16
3	22.83 ± 0.55	23.23 ± 1.03
4	22.26 ± 0.79	21.65 ± 1.27
5	20.63 ± 1.24	18.51 ± 1.11
6	20.26 ± 1.03	15.73 ± 1.46
7	20.73 ± 0.88	13.20 ± 1.83
8	19.89 ± 0.82	7.30 ± 2.12
9	18.76 ± 1.13	5.85 ± 1.46
10	18.60 ± 0.70	4.87 ± 0.56

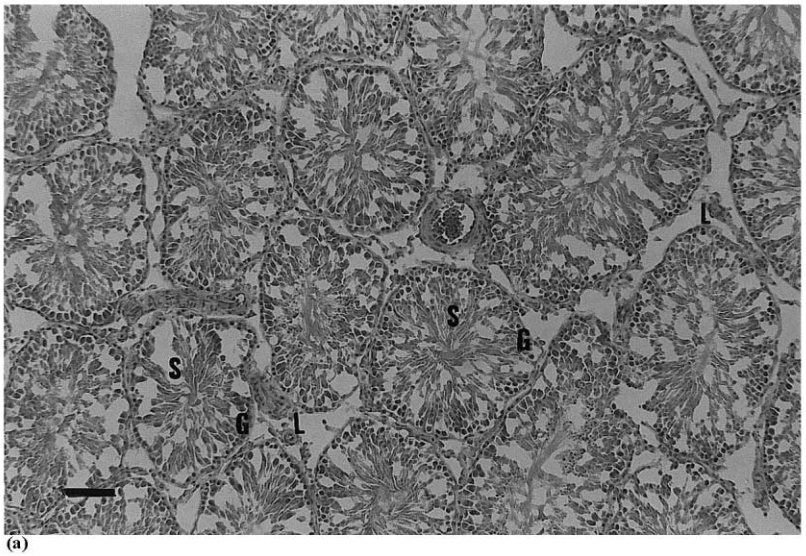
Immunisation was administered in study weeks 1, 3, 5, 7. Significant reductions in width ($P < 0.01$), compared with non-treated control occurred after the third immunisation (ANOVA, followed by Student–Newman–Keuls test).

spermatogenic cells of the tubules appeared to become degenerated and were continuously cast off. The inner wall of the tubules contained only a small number of spermatogenic cells (spermatogonium and spermatocytes) and Sertoli cells, with no visible spermatozoa under light microscopy (Fig. 3b). The inter tubular spaces were distended and contained smaller number of darkly stained spindle cells, resembling primitive gonadal stromal cells. Leydig cells lying in between the seminiferous tubules were virtually non-existent and rarely in small clusters, leaving only a few irregular polyhedral

Table 3
The mean weight and S.D. of reproductive organs at terminal sacrifice

Treatment	Organ weight ± S.D. (g)		
	Combined testes	Combined epididymis	Seminal vesicles
None	3.98 ± 0.24	1.39 ± 0.07	3.19 ± 0.19
des 1-GnRH-I-TT	0.73 ± 0.03	0.32 ± 0.01	0.30 ± 0.03

Animals were immunised against des-1 GnRH-I-TT, compared with no treatment. There was a significant reduction in organ weight ($P < 0.01$), following treatment (ANOVA, followed by Student–Newman–Keuls test). The mean body weight of the animals was 488.8 ± 27.4 g (untreated controls) and 465.0 ± 51.1 g (des-1 GnRH-I analogue treated group). There was no significant difference in body weight.



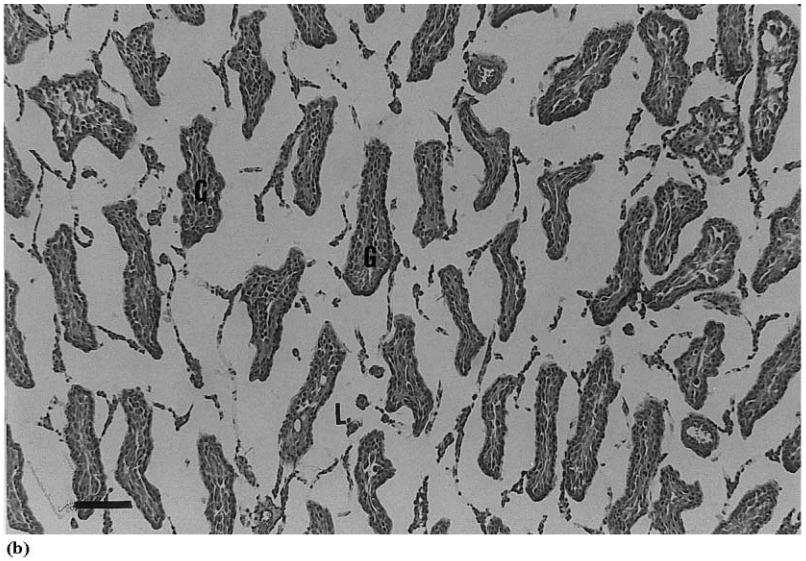
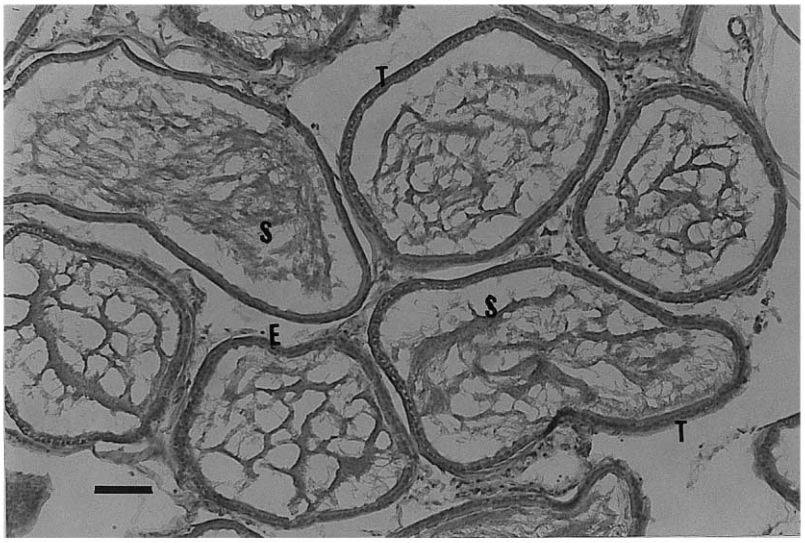


Fig. 3. Comparative sections through reproductive organs from untreated and animals immunised with des-1 GnRH-I-TT: (a) untreated rat testes, (b) treated animal testes [all photographs were taken at the same magnification (× 12.5)]. Note the presence of spermatazoa (S), germ cells (G) and Leydig cells (L). Magnification bar = 40 μm], (c) untreated epididymis head, (d) treated epididymis head, (e) untreated epididymis tail, (f) treated epididymis tail. All photographs were taken at the same magnification (× 12.5). Note the extent of distention of the spermatic tubules (T), absence and presence of spermatazoa (S), cuboidal epithelial cells (E) and columnar epithelial cells (C). Magnification bar = 40 μm.



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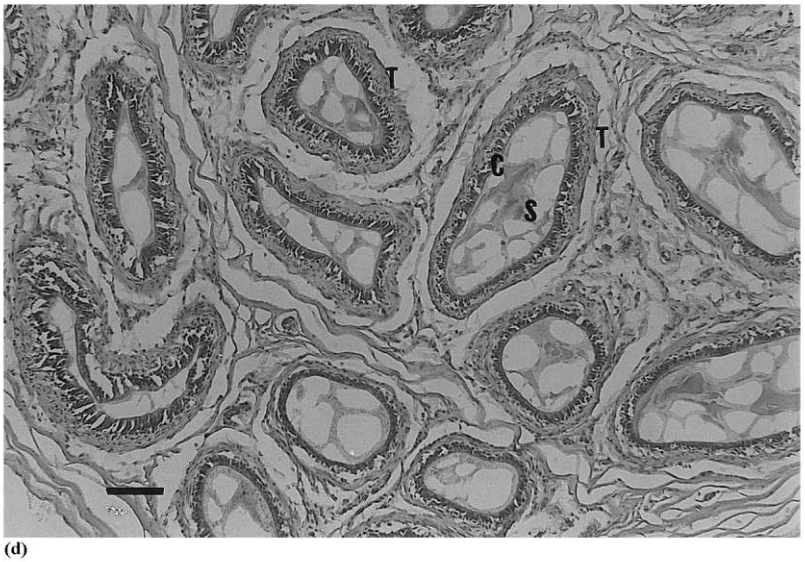
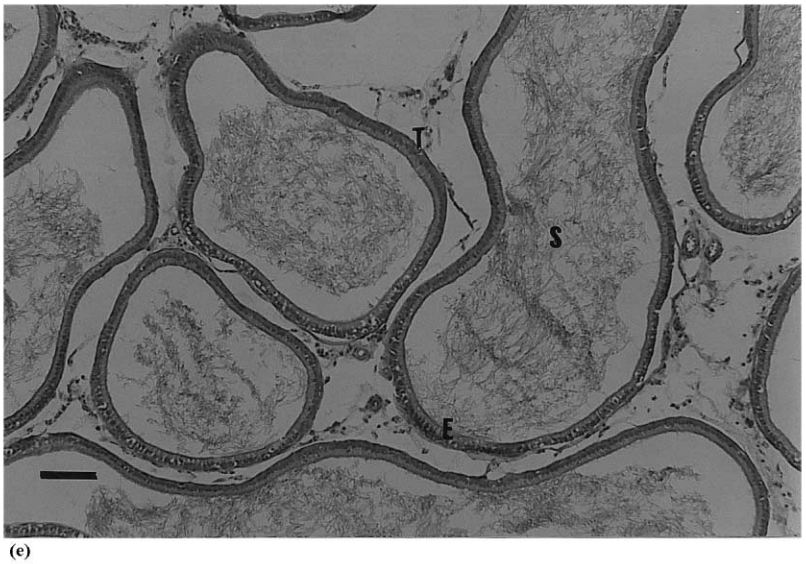


(d)

Fig. 3. (*Continued*)

cells in rows together with blood vessels. In comparison, the untreated control testis contained functionally active seminiferous tubules, lined with complex stratified epithelium (Fig. 3a) and composed of two major categories of cells (spermatogenic and Sertoli cells).



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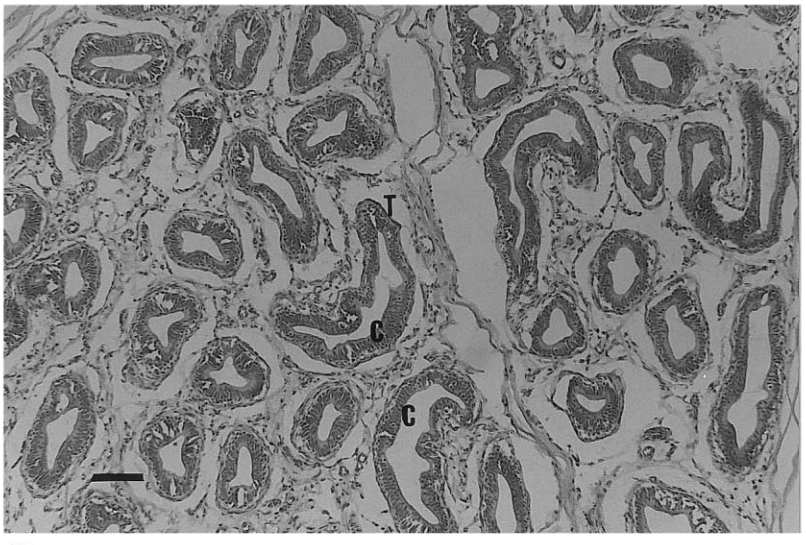

(f)

Fig. 3. (*Continued*)

The most striking changes were observed in the excretory ducts of the testes, with hyperplasia of the inner lining epithelial cells in the head, body and tail of the epididymis, which appeared to be folded. The pseudostratified columnar epithelial cells of the head of epididymis (ductuli efferentes) appeared as stratified tall columnar epithelial cells (Fig. 3c and d),

Table 4
Morphometeric analysis of seminiferous tubules from GnRH-immunized rats

	n	Mean tubule diameter (μm)	Mean tubule area (μm^2)
Control # 1	106	188 ± 1.2	$31{,}769 \pm 378$
GnRH-I # 1	83	$86 \pm 1.5^{a,b}$	$8{,}976 \pm 144^{a,b}$
GnRH-I # 2	87	$86 \pm 1.6^{a,b}$	$9{,}137 \pm 162^{a,b}$
GnRH-II # 1	106	171 ± 1.0^{a}	$26{,}655 \pm 306^{a}$
GnRH-II # 2	101	176 ± 1.1^{a}	$27{,}793 \pm 288^{a}$

Two immunised animals in each group, which showed the highest antibody levels at sacrifice were chosen for the analysis. Each value represents the mean ± S.E.M. of 83–106 seminiferous tubules.

[a] $P < 0.01$, relative to non-immunized control;

[b] $P < 0.01$, relative to GnRH-II-immunized animals (ANOVA, followed by Student–Newman–Keuls test).

with a reduced concentration of spermatozoa in the lumen of the ductuli efferentes. The smooth muscle lining the ductuli efferentes showed a thickening and similar changes were also observed in the smooth muscle layer (lining epithelium of the tail) of epididymis (Fig. 3e and f). The thin columnar epithelial cells of the tail of epididymis appeared as a stratified layer of columnar cells and the concentration of spermatozoa in the tubules was also negligible. A varying degree of loose connective tissue surrounded the tubules, together with a few infiltrated reactive cells (with the staining method used, these could not be clearly identified, but were likely to be lymphocytes or macrophages).

Histological assessment of the tubules from GnRH-II immunised animals showed abundant spermatozoa in the epididymes. However, morphometric analysis (Table 4) showed that there was significant reduction in size relative to the untreated control animals. As expected, the GnRH-I immunised animals showed that immunisation had a profound, suppressive effect.

3.7. GnRH-I and GnRH-II distribution in primate brain

Using the GnRH-I and GnRH-II antisera to stain hypothalamic sections, GnRH-I immunopositive soma and fibres were detected in the ventral hypothalamus—characteristically, the soma were fusiform and the fibres had pronounced varicosities. Rostrally, the soma and fibres were distributed in a diffuse pattern and caudally were oriented towards the median eminence (Fig. 4). In contrast, GnRH-II immunopositive fibres were densely distributed in the supraoptic nucleus and paraventricular nucleus, and caudally were densely distributed parallel to the ventral edge of the hypo-

thalamus (Fig. 4). Few, if any, GnRH-II immunopositive soma were detected. Overall, there appeared to be no overlap between the dense GnRH-II fibres and the scattered GnRH-I fibres.

4. Discussion

Immunisation against GnRH has been widely studied (Ferro and Stimson, 1999) over the past three decades, for its potential in disrupting the fertility axis. Until recently, it was believed that only one GnRH molecule was responsible in mammals for controlling sex hormone production in males and females (Conn et al., 1987). However, increasingly, there has been evidence to suggest that several forms of GnRH exist in mammals (Sherwood et al., 1993), including primates (Powell et al., 1994; Kasten et al.,

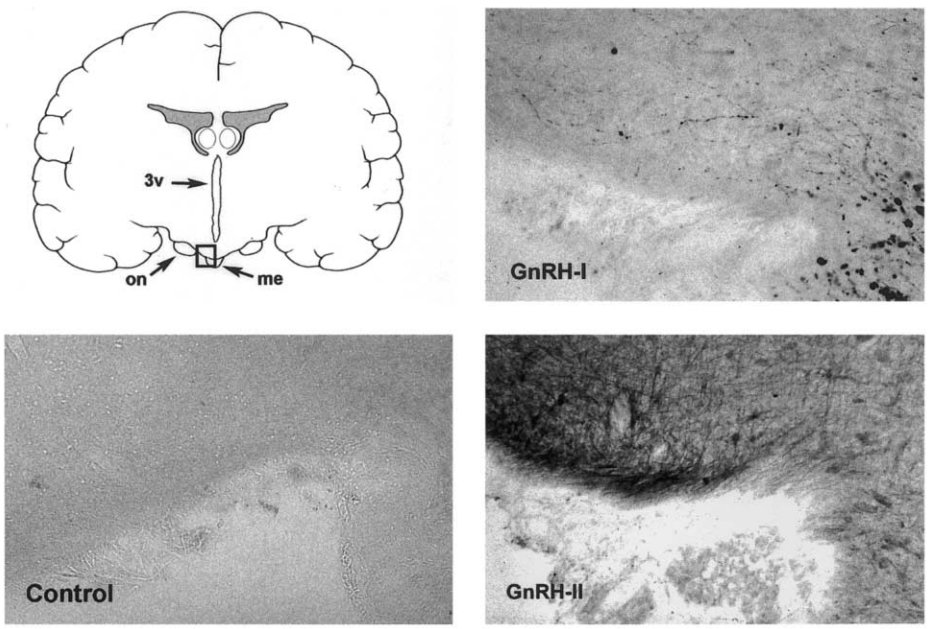

Fig. 4. Immunohistochemistry showing differential staining of neuronal fibres in the rhesus monkey hypothalamus, using antibodies to different forms of GnRH. Coronal brain sections (25 μm) were immunostained using either serum from a non-immunized rat (untreated control) or from rats immunized against GnRH-I or GnRH-II. The photomicrographs depict GnRH neuronal fibers in the ventro-medial region of the hypothalamus. Note the characteristically beaded GnRH-I fibres and densely-staining terminals in the median eminence, and the dense GnRH-II fibers running along the ventral edge of the hypothalamus. 3v = third ventricle, me = median eminence, on = optic nerve.

1996). Due to the close similarity in sequence between the GnRH molecules, it was hypothesised that immunoneutralisation of one GnRH molecule could result in concurrent ablation of all the other forms. Without knowing the significance and role of all the GnRH molecules, it was considered essential to investigate the likelihood of this problem occurring.

Initially, we examined sequences of GnRH-I and GnRH-II with the removal of two amino acids at the N terminus (des-2). Since GnRH is poorly immunogenic, the sequences were coupled to tetanus toxoid, as used previously by us (Ferro and Stimson, 1996). The method used to conjugate the carrier protein and peptides involved the use of EDC. The results showed that this type of conjugation was not efficient-therefore commercially, a large quantity of peptide would be lost in the manufacturing process; GnRH-II resulted in even lower peptide:carrier ratios. When developing the improved GnRH-I formulation, conjugation efficiency was taken into account and a cysteine residue was attached to enable the use of S-MBS, a more specific coupling agent (Aithal et al., 1988; Ferro and Stimson, 1998). The loss of peptide, using this method, was negligible.

There did not appear to be a correlation between the peptide:carrier ratio and the level of specific antibody raised. Despite the lower ratio with des-2 GnRH-II, the animals immunised with this conjugate showed slightly higher antibody levels than the des-2 GnRH-I immunised animals. However, these were surpassed by the des-1 GnRH-I analogue treated animals. The difference between these two experiments was that the des-2 animals were immunised weekly, as opposed to fortnightly in the des-1 animals. The increased IgG levels in the des-1 treated animals were not considered to be attributable to the difference in immunisation regime, but more likely to be due to the peptide:carrier ratio. Nevertheless, the pattern of response was similar in all treatment groups.

There was a low level (background) of antibody cross-reactivity in the des-2 treated animals. However, this was eliminated with the des-1 GnRH-I analogue formulation where there was negligible cross-reactivity; one reason for this could be that the antibodies formed against GnRH-I were against the hairpin loop. With the removal of the two amino acids, the hairpin loop becomes an open structure. As yet there is no information indicating what the structure for GnRH-II may be. Nevertheless, we believe from the amino acid sequence and charges present, that GnRH-II is likely to be a more open structure (as opposed to a tight hairpin). Therefore, 'structural' antibodies produced against des-2 GnRH-I are more likely to cross-react with des-2 GnRH-II, compared with any 'structural' antibodies formed against des-1 GnRH-I and vice versa.

In view of the fact that the des-1 GnRH-I antisera showed higher specificity to GnRH-I, we examined the antibody subclasses produced. It is

known that certain subclasses enable better immunocastration (Desmukh et al., 1994). The main subclasses produced were IgG2c, IgG1, IgG2a and to a lesser degree IgG2b and IgM. In a previous study (Ferro et al., 1995b), using another GnRH-I analogue, GnRH-glycys, at an equivalent concentration of peptide, the major subclasses were IgG2a, IgG1 and IgG2c, with no discernable IgM or IgG2b. However, it should be noted that the carrier protein in this study was PPD (purified protein derived from tuberculin) and the adjuvant was Freund's Adjuvant. These factors may have had an influence on the subclasses produced. Little is known about the subclasses and the criteria for their production in the rat, however, it is believed that IgG1 and IgG2a indicate that both humoral and cellular immunisation was achieved (Philips et al., 1999). The role of IgG2c is thought to be T cell independent, and the antibodies tend to be of low affinity (Peters et al., 1999). Therefore, we believe that the critical subclasses in the present context are in fact IgG1 and IgG2a.

The effect of the neutralising antibody levels was observed in vivo as a reduction in testicular size. Many studies have shown that the physical changes in the gonads requires a critical antibody titre together with a reduction in testosterone levels (Schally and Schally, 1987; Adams and Adams, 1990). In our study, optimum antibody levels (achieved by study week 3, following the second immunisation) corresponded with a significant decrease in testicular width, with a 2-week lag-period (study week 5). This corresponded to our experience with the analogue GnRH-glycys (Ferro and Stimson, 1996), whereby a decrease in testosterone levels was shown to occur following the second administration of immunogen and gonadal atrophy began a week later. The reduction in testicular size continued until the end of the study (a period of 6 weeks).

Numerous studies (Awoniyi et al., 1989; Hoskinson et al., 1990; Giri et al., 1991; Dowsett et al., 1993; Ladd et al., 1994; Meloen et al., 1994; Kumar et al., 2000), in a wide range of animal species, have described the effect of active immunisation against GnRH-I on the gonads and accessory organs. Morphological changes in the gonads were examined at the end of each study. In this study we examined the testes, epididymes, prostate and seminal vesicles. The des-2 GnRH-II treated animals did not show any gross alteration in the gonads or accessory sex organs. However, they showed a slight increase in kidney weight (results not shown) which may be indicative of a role for GnRH-II in osmoregulation. Histological assessment showed drastic changes in testicular morphology, as well as atrophy to the seminiferous tubules and a total depression in spermatogenesis in the GnRH-I immunised animals. From other GnRH-neutralisation studies (Ferro et al., 1995a; Ferro and Stimson, 1996), it has been shown that these effects are reversible after a few months. Histologically, examination of the

testicular tissue and excretory ducts have indicated a clear manifestation of secondary infertility. The hypogonadal state caused the dysfunction of the Leydig and Sertoli cells of the testes following neutralisation of GnRH. Although there is no evidence of spermatogenesis in the tubules of the testes, very few spermatozoa were seen in the head of epididymis and their presence was not found in the tail of the epididymis. Androgen action is necessary for spermatogenesis in seminiferous tubules and for epididymal function, but it has been noted that it is especially important for the maturation of spermatozoa in the tail of epididymis (Wilson and Foster, 1992).

Although spermatogenesis was apparently not affected by GnRH-II immunisation (results not shown), morphometric analysis of the seminiferous tubules indicates that there is a slight reduction on tubule size.

In order to further investigate and elucidate a role for GnRH-II, the GnRH-II antisera was used to immunostain primate hypothalmic sections. The deduction from this work was that GnRH-I and GnRH-II are produced by two distinct populations of neurones and it is likely that the synthesis and release of these two peptides are regulated by different neuroendocrine pathways. This corroborates results from other studies (Lescheid et al., 1997) using in situ hybridisation, which implicate the same hypothalamic regions in GnRH-II expression. In this study, a possible function in reproduction is indicated for GnRH-II, as well as a role associated with the kidney. GnRH-II neurones were not found to overlap in location with GnRH-I neurones. Further work is required to investigate co-localisation of GnRH-II secreting cells with other endocrine cells, such as oxytocin and vasopressin secretors (Latimer et al., 2000). Nevertheless, studies have shown that GnRH-II stimulates LH release in vivo (Lescheid et al., 1997) and one possibility is that GnRH-II is important as a pulse generator which may stimulate the onset of puberty (Latimer et al., 1999, 2000). Therefore, immunoneutralisation in the adult animal may not be relevant.

This study demonstrated an effective means of specifically immunoneutralising related peptide sequences, without invoking cross-reactivity. In addition, an analogue was designed which could immunocastrate male animals, without any inappropriate side effects. This vaccine will be further developed for use in clinical and veterinary applications.

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